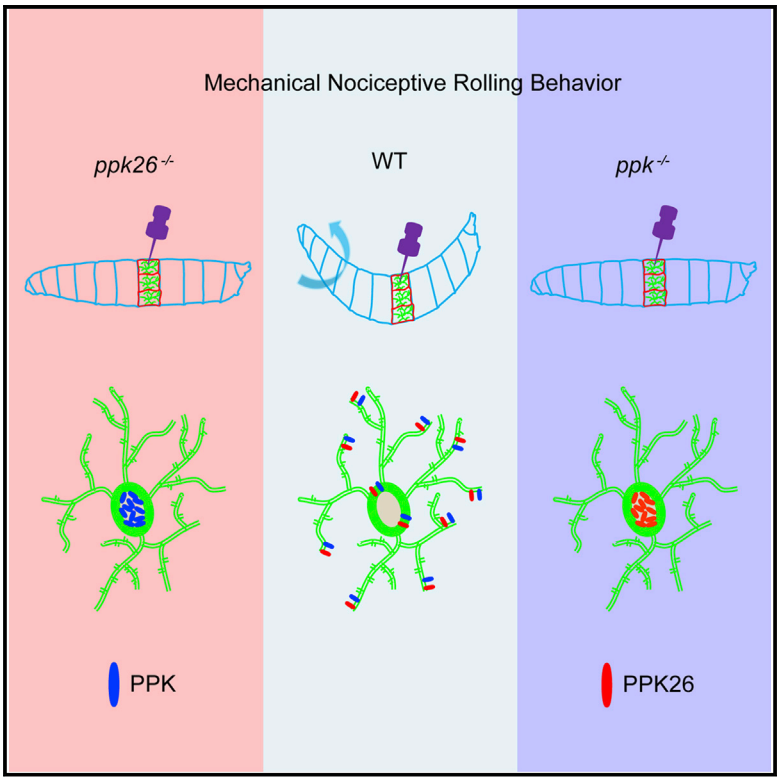
Report

Cell Reports

The Role of PPK26 in *Drosophila* Larval Mechanical Nociception

Graphical Abstract



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In Brief

Sensing painful stimuli is of vital importance for animal survival. Guo et al. now find that PPK26 is selectively expressed in class IV dendritic arborization neurons and contributes to mechanical nociception but not thermal nociception in *Drosophila* larvae.

Highlights

PPK26 is specifically expressed in class IV da neurons in *Drosophila* larvae

PPK26 participates in mechanical nociception but not in thermal nociception

PPK26 and PPK function in the same pathway in mechanical nociception

PPK and PPK26 are interdependent on each other for their plasma membrane localization

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Report

The Role of PPK26 in *Drosophila* Larval Mechanical Nociception

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SUMMARY

In *Drosophila* larvae, the class IV dendritic arborization (da) neurons are polymodal nociceptors. Here, we show that *ppk26* (CG8546) plays an important role in mechanical nociception in class IV da neurons. Our immunohistochemical and functional results demonstrate that *ppk26* is specifically expressed in class IV da neurons. Larvae with mutant *ppk26* showed severe behavioral defects in a mechanical nociception behavioral test but responded to noxious heat stimuli comparably to wild-type larvae. In addition, functional studies suggest that *ppk26* and *ppk* (also called *ppk1*) function in the same pathway, whereas *piezo* functions in a parallel pathway. Consistent with these functional results, we found that PPK and PPK26 are interdependent on each other for their cell surface localization. Our work indicates that PPK26 and PPK might form heteromeric DEG/ENaC channels that are essential for mechanotransduction in class IV da neurons.

INTRODUCTION

Mechanical sensations are of vital importance for animals to coordinate their behaviors. Many physiological processes, including proprioception, hearing, touch sensation, and pain, depend on the conversion of mechanical forces into ion currents (Chalfie, 2009). Although widely studied, the molecular mechanisms underlying mechanotransduction still remain to be fully understood. Mechanotransduction is directly mediated by mechanogated ion channels, because the responses of mechanoreceptors to forces are too rapid to involve a second-messenger cascade (Corey and Hudspeth, 1979; O'Hagan et al., 2005; Walker et al., 2000). To date, only a few members of the DEG/ENaC, TRP, Piezo, and TMC families have been demonstrated to be mechanosensitive ion channels in the nervous system (Chatzigeorgiou et al., 2010; Coste et al., 2012; Geffeney et al., 2011; Gong et al., 2013; Kang et al., 2010; Kim et al., 2012; O'Hagan et al., 2005; Pan et al., 2013; Yan et al., 2013).

In response to noxious stimuli, animals usually perform stereotyped avoidance or withdrawal behaviors, suggesting that nociceptive stimuli are processed by evolutionarily conserved mechanisms that are crucial for avoiding potential tissue damage. It is therefore not surprising that animals ranging from worms and fruit flies to mammals have evolved dedicated pain-sensing nociceptors that cover the entire body surface. In mammals, noxious stimuli are sensed by high-threshold mechano-nociceptors and polymodal nociceptors, which respond to stimuli of more than one modality (Basbaum et al., 2009). The class IV dendritic arborization (da) neurons represent the polymodal nociceptors in *Drosophila* larvae (Hwang et al., 2007). They detect intense mechanical forces, noxious heat, harmful short-wave light, and dry-surface environments, as well as harmful hydrogen peroxide (Hwang et al., 2007; Johnson and Carder, 2012; Kim et al., 2013; Kim and Johnson, 2014; Xiang et al., 2010). The PPK, Pain (also called Painless), and Piezo proteins all contribute to mechanical nociception in class IV da neurons (Kim et al., 2012; Tracey et al., 2003; Zhong et al., 2010, 2012). However, the roles of DEG/ENaC channels other than PPK in mechanical nociception have remained largely unknown. Here, we report that *ppk26* is selectively expressed in larval class IV da neurons, where it contributes to mechanical, but not thermal, nociception along with *ppk*.

RESULTS

Specific Expression of PPK26 in Class IV da Neurons

To date, 31 members of the DEG/ENaC channel family have been identified in the *Drosophila melanogaster* genome (Zelle et al., 2013). Although several have been shown to participate in taste sensation and courtship (Ben-Shahar, 2011), only PPK and Ripped Pocket (RPK) have been implicated in mechanosensation (Tsubouchi et al., 2012; Zhong et al., 2010). To investigate whether other PPK family channels might be involved in mechanotransduction, we first performed a multiple sequence alignment and constructed a phylogenetic tree of all identified *Drosophila* DEG/ENaC proteins using the COBALT program (Papadopoulos and Agarwala, 2007). Similar to the findings of a previous report (Zelle et al., 2013), the phylogenetic analysis indicated that PPK, RPK, PPK5, PPK8, PPK12, PPK26, and PPK28 might form a subfamily (data not shown). Basing on the function of PPK and RPK (Tsubouchi et al., 2012; Zhong et al.,

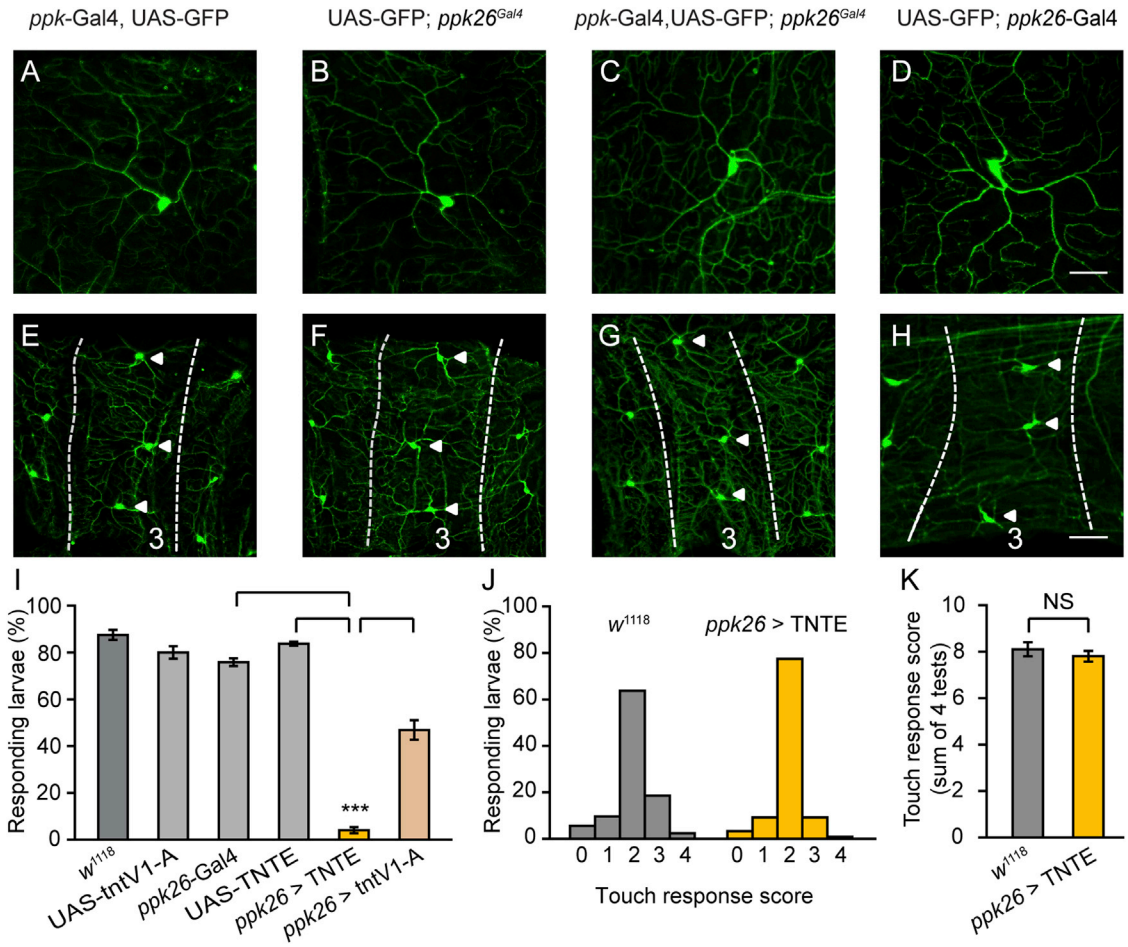

Figure 1. Specific Expression of *ppk26*^{Gal4} and *ppk26*-Gal4 in Class IV da Neurons

(A–H) Morphology (A–D) and neuronal number per hemisegment (E–H) of larvae with the indicated genotypes. (A–D) The scale bar represents 50 μM. (E–H) The scale bar represents 100 μM.

(I) Effect of blocking synaptic transmission of *ppk26*-positive neurons (*ppk26* > TNTE) on rolling behavior in the 47 mN mechanical nociception assay. For each genotype, at least four independent trials were performed, with 40 larvae in each trial. One-way ANOVA was used to test the differences between *ppk26*-Gal4, UAS-TNTE, and *ppk26* > TNTE ($p < 0.05$), followed by Scheffe's post hoc test. Two-tailed unpaired Student's t test was used to test the difference between *ppk26* > TNTE and *ppk26* > tntV1-A. ***$p < 0.001$.

(J and K) Score distribution (J) and statistical analysis (K) of gentle touch responses in wild-type (w^{1118}) and *ppk26* > TNTE larvae. $n \geq 30$. NS, not significant; $p = 0.48$. Two-tailed unpaired Student's t test.

Error bars indicate SEM.

See also Figures S1 and S3.

2010), we reasoned that the other PPKs in this subfamily may also be involved in mechanosensation, and we therefore investigated them further. We first examined the expression patterns of *ppk5*, *ppk12*, and *ppk28* by using *ppk5*-Gal4, *ppk12*-Gal4, and *ppk28*-Gal4 to drive upstream activating sequence (UAS)-mCD8-GFP expression. We did not observe any detectable expression of GFP in potential mechanosensitive multidendritic neurons (data not shown). On the basis of the phylogenetic analysis and the expression results, we excluded PPK5, PPK12, and PPK28 from further studies on mechanosensation. Because PPK, RPK, and PPK26 are close paralogs, we examined their expression patterns and potential roles in mechanosensation. First, we generated reporter alleles of *rpk* and *ppk26* by end-out homologous recombination (Gong and Golic, 2003; Moon et al., 2009). The *Gal4* transcription activator gene was inserted into the genomic loci of *rpk* and *ppk26*, near the site of the normal translation initiation codon (Figure S1). The *rpk* and *ppk26* reporter alleles were named *rpk*^{Gal4} and *ppk26*^{Gal4}, respectively. By examining the reporter-expression pattern in larvae using the GAL4/UAS system, we did not observe any obvious expression of *rpk*^{Gal4} in larval da neurons. In contrast, *ppk26*^{Gal4} was specifically expressed in class IV da neurons, which we identified on the basis of the morphology of the mCD8-GFP-positive neurons (Figures 1A–1C). The *ppk26*^{Gal4} allele is a *ppk26*-null mutant because a portion of the coding sequence, including the N terminus and the first transmembrane motif, is deleted (Figure S1).

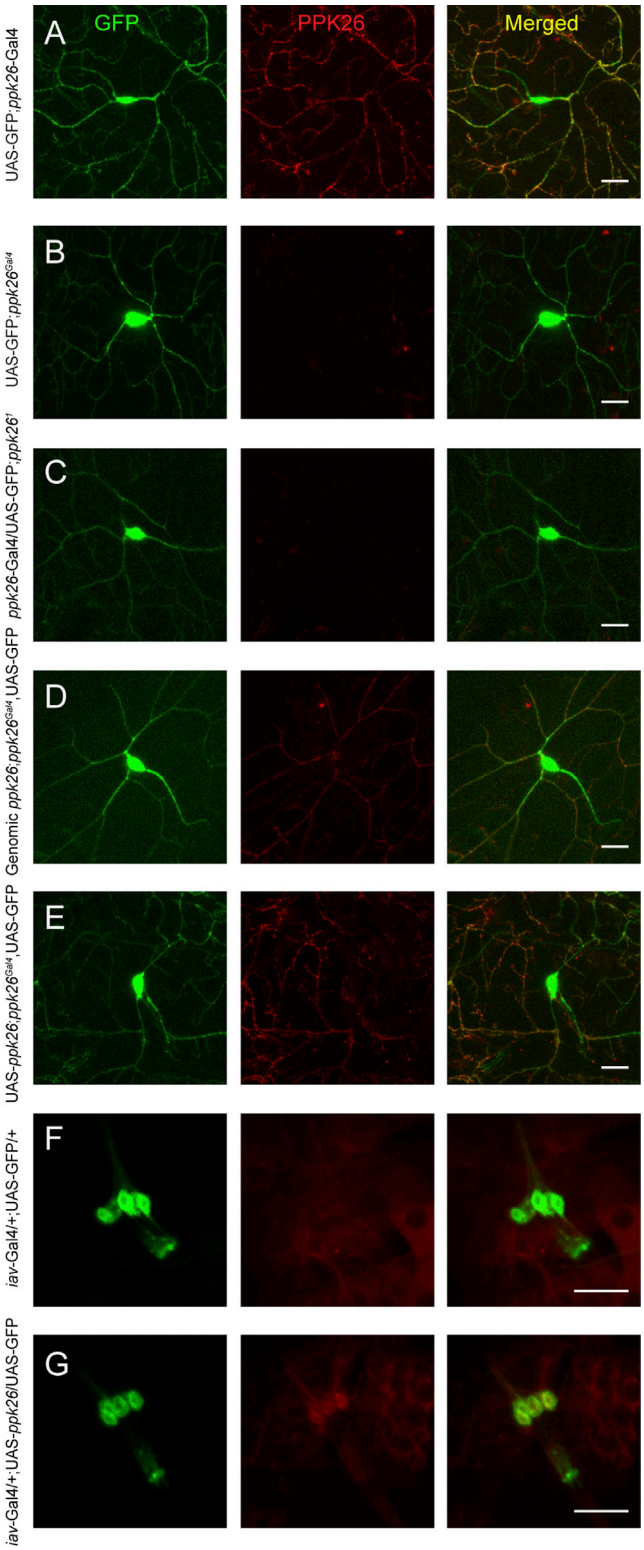

Figure 2. Endogenous Expression of PPK26 in Class IV da Neurons

Immunostaining of PPK26 in *Drosophila* larvae with the indicated genotypes: (A) wild-type; (B) $ppk26^{Gal4}$-null mutant; (C) $ppk26^{1}$-null mutant; (D) genomic *ppk26* rescue; (E) specific rescue in class IV da neurons; (F) ectopic expression control; and (G) ectopic expression of *ppk26*. The scale bars represent 25 μM.

Note: the cell membrane was not permeabilized in (A)–(E), whereas it was permeabilized in (F) and (G) using Triton X-100.

See also Figure S2.

The similar morphology of $ppk26^{Gal4}$-positive neurons and class IV da neurons labeled by *ppk*-Gal4 indicated that the *ppk26* mutation did not affect the neuronal morphology. We also generated a normal transgenic Gal4 driver line of *ppk26*, *ppk26*-Gal4. Unlike the $ppk26^{Gal4}$ knockin allele, the *ppk26*-Gal4 transgene did not affect the endogenous *ppk26* gene. Similar to $ppk26^{Gal4}$, *ppk26*-Gal4 also specifically labeled class IV da neurons (Figures 1D and 1H). We found three *ppk*-Gal4-labeled class IV da neurons in each hemisegment (Figure 1E), consistent with previous reports (Grueber et al., 2002); $ppk26^{Gal4}$ also labeled exactly three neurons in each hemisegment (Figure 1F). Even when the expression of UAS-mCD8-GFP was driven by *ppk*-Gal4 and $ppk26^{Gal4}$ simultaneously, there were still three neurons in each hemisegment (Figure 1G). These results indicated that $ppk26^{Gal4}$ specifically labels class IV da neurons. We did not observe any labeling of other types of peripheral neurons or CNS neurons in fly larvae by *ppk26*-Gal4 or $ppk26^{Gal4}$ (Figures 1E–1H, S2A, and S2B).

The Neurons Labeled by *ppk26*-gal4 Were Required for Detection of Noxious Mechanical and Thermal Stimuli

The class IV da neurons, activation of which is necessary and sufficient to elicit the stereotyped rolling behavior in *Drosophila* larvae, are polymodal nociceptors (Hwang et al., 2007). Therefore, we examined the effect of blocking synaptic transmission of *ppk26*-Gal4-positive neurons on nociceptive rolling behavior with tetanus toxin light chain (TeTxLC, also called TNT; Sweeney et al., 1995). TNT was selectively expressed in *ppk26*-Gal4-positive neurons (*ppk26* > TNTE). We found that, in contrast to Gal4 alone, UAS-TNTE controls, and expression of the inactive tntV1-A form of the tetanus toxin light chain, blocking synaptic transmission of *ppk26*-Gal4-positive neurons with TNT almost totally abolished the rolling behavior of *Drosophila* larvae in response to 47 millinewton (mN) noxious mechanical forces (Figure 1I). Silencing *ppk26*-Gal4-positive neurons also significantly prolonged the response delay to 47°C noxious heat (Figure S3). All of these results are consistent with previous findings (Hwang et al., 2007). Furthermore, behavioral response to gentle touch, which is mediated by class III da neurons (Yan et al., 2013), was not affected (Figures 1J and 1K). These results further confirmed that *ppk26*-Gal4 selectively labeled class IV da neurons and did not label class III da neurons.

Endogenous Expression of PPK26 in Class IV da Neurons

To further clarify the endogenous expression pattern of *ppk26*, we performed immunohistochemical experiments using a polyclonal antibody raised against a synthetic peptide (residues 497–510) from the extracellular domain sequence of the PPK26 protein. Our immunostaining results confirmed that endogenous PPK26 protein was selectively expressed in class IV da neurons in wild-type larvae but not *ppk26* mutant larvae (Figures 2A–2C).

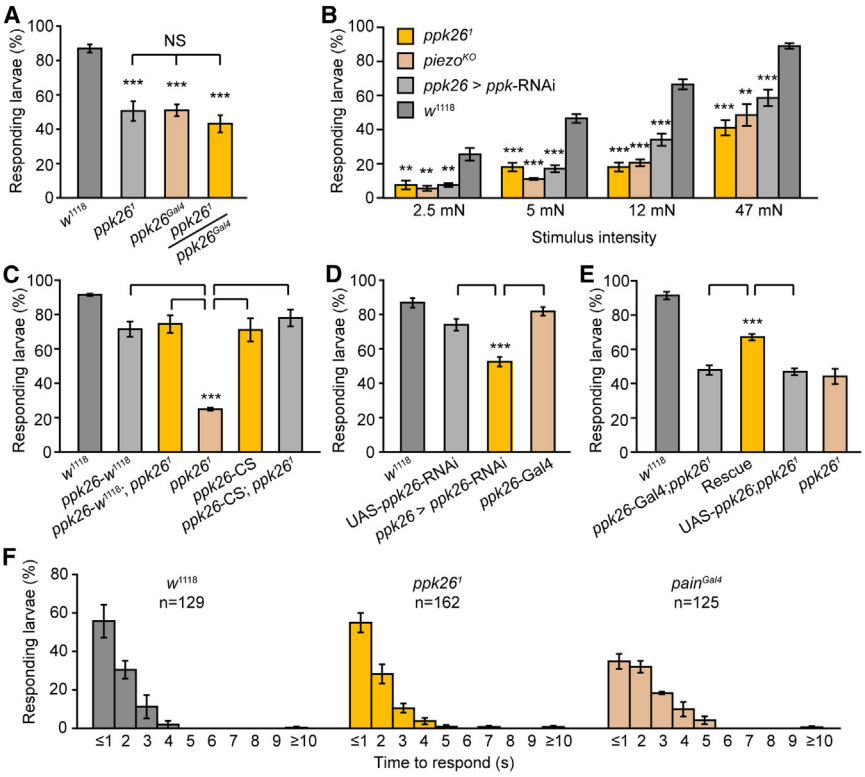

Figure 3. *ppk26* Contributes to Mechanical Nociception but Not Thermal Nociception

(A) Percentage of wild-type (w^{1118}) and *ppk26*-null mutant ($ppk26^{1}$, $ppk26^{Gal4}$, and transheterozygous mutant $ppk26^{1}/ppk26^{Gal4}$) larvae showing rolling behavior in response to 47 mN nociceptive mechanical stimuli. One-way ANOVA was used to test the differences between w^{1118} and *ppk26*-null mutants ($p < 0.05$), followed by Scheffe's post hoc test. *** indicates $p < 0.001$ between w^{1118} and corresponding null mutant. No differences were detected between *ppk26*-null mutants in one-way ANOVA analysis ($p = 0.53$).

(B) Percentage of *ppk26*-null mutant $ppk26^{1}$, *Dmpiezo*-null mutant $piezo^{KO}$, and *ppk* knockdown (*ppk26* > *ppk*-RNAi) larvae showing rolling behavior in the mechanical nociception assay over a wide range of forces. KO, knockout. One-way ANOVA was used to test the differences between w^{1118} and $ppk26^{1}$, $piezo^{KO}$, *ppk26* > *ppk*-RNAi larvae ($p < 0.05$), followed by Scheffe's post hoc test. **$p < 0.01$ and ***$p < 0.001$ between w^{1118} and corresponding larvae.

(C) Percentage of genomic *ppk26* rescue larvae exhibiting rolling behavior in the 47 mN mechanical nociception assay. One-way ANOVA was used to test the differences between genomic control (*ppk26*-w^{1118}), genomic rescue (*ppk26*-w^{1118};$ppk26^{1}$), and $ppk26^{1}$ ($p < 0.05$), followed by Scheffe's post hoc test. *ppk26*-CS control and genomic rescue was analyzed independently in the same way. ***$p < 0.001$.

(D) Percentage of larvae in which *ppk26* was specifically knocked down in class IV da neurons by *ppk26*-Gal4 and UAS-*ppk26*-RNAi that exhibited rolling behavior. ***$p < 0.001$. One-way ANOVA analysis followed by Scheffe's post hoc test.

(E) Percentage of larvae in which *ppk26* was specifically expressed in class IV da neurons in a $ppk26^{1}$ mutant background that exhibited rolling behavior in the 47 mN mechanical nociception assay. ***$p < 0.001$. One-way ANOVA analysis followed by Scheffe's post hoc test.

(F) The *ppk26*-null mutant $ppk26^{1}$ exhibited a normal response delay in the 47°C thermal nociception assay. $n \geq 125$.

For each genotype, at least four independent trials were performed. Data are shown as mean ± SEM.

See also Figure S3.

More importantly, the anti-PPK26 staining signal could be detected throughout the dendrites of class IV da neurons, whereas no obvious staining signal was observed at the axon terminals (Figures 2A and S2A). Because these immunohistochemical staining experiments were performed without permeabilizing the cell membrane, these results also indicated that the PPK26 protein is intrinsically localized on the plasma membrane. No anti-PPK26 signals could be detected in other types of peripheral neurons or CNS neurons (Figures S2A and S2C). When a genomic transgene of *ppk26* was introduced back into the $ppk26^{Gal4}$-null mutant background, the immunofluorescent signals were restored in class IV da neurons (Figure 2D).

We also generated a UAS-*ppk26* transgene for overexpression. We found that overexpressing *ppk26* in the $ppk26^{Gal4}$ mutant background by crossing $ppk26^{Gal4}$ with UAS-*ppk26* similarly restored the immunofluorescent signal (Figure 2E). In addition, when we used an *iav*-Gal4 driver to ectopically overexpress *ppk26* in chordotonal organ neurons, which normally do not express *ppk26* (Figure 2F), immunofluorescent signals were detected in these neurons (Figure 2G). However, no immunofluorescent signal could be detected if the cell membrane was not permeabilized (data not shown), suggesting that PPK26 does not translocate to the plasma membrane on its own. All of these results demonstrated that the antibody specifically recognized PPK26 and that PPK26 protein is endogenously expressed in class IV da neurons. Our immunostaining results also indicated that the $ppk26^{Gal4}$ and *ppk26*-Gal4 reporter alleles faithfully represented the expression pattern of *ppk26*.

PPK26 Is Required for Mechanical Nociception but Not Thermal Nociception

Next, we examined the physiological role of PPK26 in nociception. We first tested the function of PPK26 in mechanical nociception by examining the rolling behavior of *ppk26*-null mutant larvae in response to intense forces. We also generated another *ppk26*-null mutant allele, $ppk26^{1}$, in which the deleted coding region was identical to the region deleted in $ppk26^{Gal4}$ (Figure S1). The $ppk26^{1}$-null allele was verified by PCR and immunostaining (Figure 2C). The percentage of larvae exhibiting rolling behavior in response to 47 mN mechanical forces was remarkably reduced in $ppk26^{1}$, $ppk26^{Gal4}$, and transheterozygous $ppk26^{1}/ppk26^{Gal4}$ larvae relative to wild-type w^{1118} larvae (Figure 3A). In addition, transheterozygous $ppk26^{1}/ppk26^{Gal4}$ larvae exhibited reduction in rolling behavior comparable to those in

ppk26^{Gal4} larvae and *ppk26*^{1} larvae (Figure 3A), suggesting that the behavioral deficit in mechanical nociception was indeed caused by the *ppk26* mutation. Previous studies showed that *pain*, *ppk*, and *piezo* all participate in mechanical nociception in class IV da neurons (Kim et al., 2012; Zhong et al., 2010, 2012). We found that *ppk26* mutation, *piezo* mutation, and knockdown of *ppk* produced reductions in rolling behavior in response to a wide range of forces (Figure 3B), indicating that all of these channels contributed to the sensation of forces of different intensity. Furthermore, the behavioral defects caused by *ppk26* mutation could be rescued by introducing a *ppk26* genomic transgene back into the *ppk26*^{1} mutant (Figure 3C). All of these results indicated that *ppk26* contributed to mechanical nociception. Then we asked whether specific expression of *ppk26* in class IV da neurons is important for mechanical nociception. We found that specific knockdown of *ppk26* in class IV da neurons using *ppk26*-Gal4 also significantly decreased the rolling behavior in response to 47 mN mechanical forces (Figure 3D). The behavioral defects in the *ppk26* mutants in the mechanical nociception assay were rescued by selective expression of *ppk26* in class IV da neurons using *ppk26*-gal4 (Figure 3E). All of these results suggest that the function of *ppk26* in class IV da neurons is essential for mechanical nociception. Next, we examined the function of *ppk26* in thermal nociception. We found that, in contrast to silencing the *ppk26*-Gal4-positive neurons (Figure S3), *ppk26* mutants showed a response delay to 47°C noxious heat comparable to that of wild-type larvae (Figure 3F), indicating that *ppk26* does not participate in thermal nociception. Importantly, the specific behavioral deficit of *ppk26* mutants in mechanical nociception, but not thermal nociception, indicated that the *ppk26* mutation did not impair the ability of the larvae to produce rolling behavior. It also suggests that *ppk26* might directly participate in mechanical transduction, rather than regulating the neuronal development or intrinsic properties of class IV da neurons.

PPK26 and PPK Function in the Same Pathway

Two parallel pathways underlie larval noxious mechanotransduction in class IV da neurons (Kim et al., 2012). *piezo* and *pain* work in the same pathway in noxious mechanosensation, whereas *ppk* functions in a parallel pathway (Kim et al., 2012). Blocking synaptic transmission of class IV da neurons almost completely abolished the rolling behavior elicited by noxious mechanical stimulation (Figure 1I), whereas mutation of *ppk26* only caused a moderate behavioral deficit (Figure 3A), suggesting that *ppk26* might participate in only one pathway. We used a genetic interaction strategy to investigate which pathway *ppk26* participates in. The *ppk26* and *piezo* double mutant showed a more-severe behavioral defect than either single mutant, suggesting that these two proteins might function in parallel pathways (Figures 4A and 4B). On the contrary, the *ppk26*^{1} and *ppk* double mutant showed a comparable behavioral defect to the *ppk26*^{1} single mutant (Figure 4A). Moreover, after we backcross *ppk26*^{Gal4} with *w*^{1118} flies, the *ppk26*^{Gal4} and *ppk* double mutant showed a comparable behavioral defect to each of the single mutants (Figure 4B). All of these results suggest that *ppk26* and *ppk* might work in the same pathway. Using the PPK26 antibody mentioned above, we also examined the effect of *ppk* mutation on the membrane localization of PPK26. In wild-type larvae, the PPK26 antibody clearly stained the cell surface (Figure 4C). We detected no obvious PPK26 antibody staining in *ppk* mutant larvae (Figure 4D) as long as we did not permeabilize the cell membrane, suggesting that *ppk* is required for the translocation of PPK26 to the plasma membrane. However, the morphology of class IV da neurons and the expression of *ppk26* were not affected by *ppk* mutation (Figures S4A–S4D). In contrast to the results in *ppk* mutant larvae, PPK26 antibody staining was detected on the cell surface in *piezo* mutant larvae (Figure 4E), suggesting that the *piezo* mutation did not affect PPK26 surface localization. In the same way, we found that *ppk26*, but not *piezo*, is required for the plasma membrane localization of PPK (Figures 4F–4I). Likewise, the morphology of class IV da neurons and the expression of *ppk* were not affected by *ppk26* mutation (Figures 1A, 1B, and S4E–S4G). Because PPK and PPK26 both belong to the DEG/ENaC family, whose members form channels with three homogeneous or heterogeneous subunits (Geffeney and Goodman, 2012), they are likely to form heterogeneous DEG/ENaC channels.

DISCUSSION

The similar cellular functions and behavioral outputs of class IV da neurons in fruit flies and polymodal nociceptors in *C. elegans* and mammals suggest that they may share similar molecular mechanisms. Our findings confirmed that the DEG/ENaC channels PPK26 and PPK participate in mechanical nociception in *Drosophila* larvae. In addition, DEG/ENaC channels are required for response to harsh touch in *C. elegans* (Chatzigeorgiou and Schafer, 2011; Chatzigeorgiou et al., 2010; Delmas and Coste, 2013; Geffeney et al., 2011; Li et al., 2011). The role of mammalian DEG/ENaC channels in mechanical nociception remains unclear, because no consistent behavioral defects have been observed in genetic knockout mice (Basbaum et al., 2009), and need to be further clarified. Our genetic interaction experiments and surface expression experiments indicated that PPK26 and PPK might translocate to the plasma membrane with each other after they interact and they might form heteromeric channels. The relationship between acid-sensing ion channels (ASICs) in DRG neurons should also be examined to clarify whether, like PPK and PPK26, they are interdependent for their surface expression and whether they form heteromeric channels or work redundantly in mechanical nociception.

Unlike *ppk* and *ppk26*, we found that *piezo* and *ppk26* work in parallel pathways (Figure 4A). It is reasonable to wonder why mechanical nociception involves two pathways: the *ppk* pathway and *piezo* pathway. One possibility is that the two different pathways might respond to forces of different intensity. However, the results of our behavioral experiments do not support this idea, because mutation of either pathway caused behavioral defects in response to a wide range of forces (Figure 3B). Another possibility, although we do not have data to support it, is that the sensations of noxious mechanical stimuli are so vital that animals have evolved two redundant pathways to increase survival and provide an evolutionary advantage.

There is still no clear evidence that mechanogated ion currents can be detected when DEG/ENaC channels are ectopically

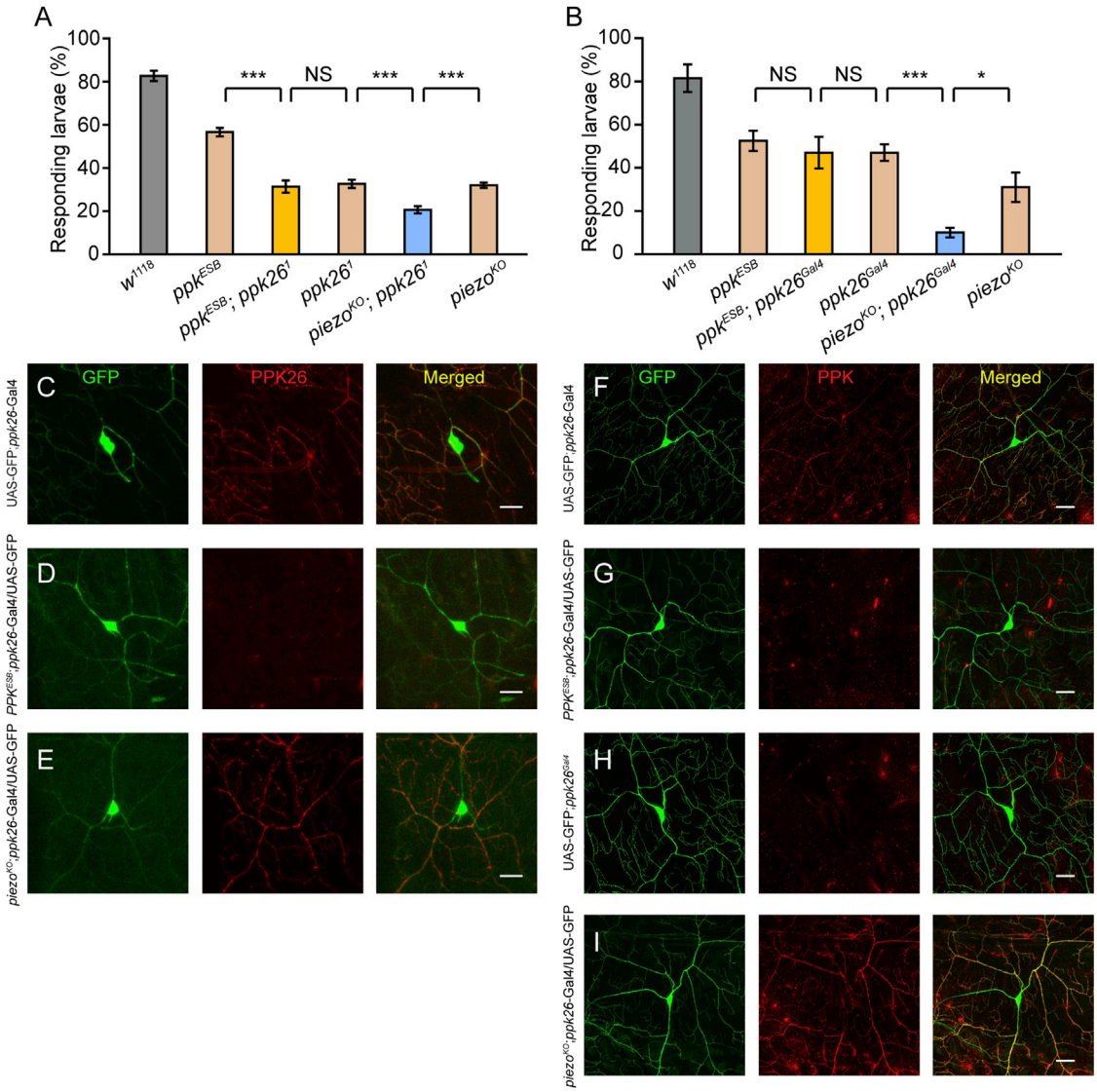

Figure 4. PPK and PPK26 Function in the Same Pathway

(A) The percentage of *ppk* and *ppk26*^{1} double mutants and *piezo* and *ppk26*^{1} double mutants exhibiting rolling behavior in the 45 mN mechanical nociception assay. For each genotype, 20 independent trials were performed. One-way ANOVA was used to test the differences between *ppk* and *ppk26*^{1} double mutants and corresponding single mutants ($p < 0.05$), followed by Scheffe's post hoc test. Differences between *piezo* and *ppk26*^{1} double mutants and corresponding single mutants were analyzed independently in the same way. ***$p < 0.001$. Data are represented as mean ± SEM.

(B) The percentage of *ppk* and *ppk26*^{Gal4} double mutants and *piezo* and *ppk26*^{Gal4} double mutants exhibiting rolling behavior in the 45 mN mechanical nociception assay. For each genotype, five independent trials were performed. One-way ANOVA was used to test the differences between *ppk* and *ppk26*^{Gal4} double mutants and corresponding single mutants ($p = 0.72$). Differences between *piezo* and *ppk26*^{Gal4} double mutants and corresponding single mutants were analyzed independently in the same way. *$p < 0.05$; ***$p < 0.001$. Data are represented as mean ± SEM.

(C–E) Immunostaining of PPK26 in *Drosophila* larvae with the indicated genotypes: (C) wild-type; (D) *ppk*-null mutant; and (E) *piezo*-null mutant.

(F–I) Immunostaining of PPK in *Drosophila* larvae with the indicated genotypes: (F) wild-type; (G) *ppk*-null mutant; (H) *ppk26*-null mutant; and (I) *piezo*-null mutant.

Note: the cell membrane was not permeabilized in (C)–(I).

The scale bars represent 25 μM.

See also Figure S4.

expressed in heterogeneous systems (Arnadóttir and Chalfie, 2010). We have ectopically coexpressed PPK and PPK26 in human embryonic kidney 293T and *Drosophila* S2 cells, but unfortunately, no obvious mechanosensitive ion currents were recorded from these cells (data not shown). We have also performed in situ whole-cell patch recording on larval class IV da neurons but did not detect any mechanosensitive ion currents in response to mechanical displacements toward the dendritic area of up to 100 μM (data not shown). It is possible that they are pore-forming subunits but that they need much larger

mechanical forces to activate than that we have given, because nociceptors are usually activated with a high threshold. PPK has also been reported to be activated by acid (Boiko et al., 2012), similar to mammalian ASICs. It is possible that DEG/ENaC channels might be activated indirectly by acids in mechanical nociception. The gating mechanism of DEG/ENaC channels in mechanotransduction is still not fully understood, and more thorough studies are needed to address this question.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

The following flies were used: *ppk*^{ESB} (Boiko et al., 2012); *piezo*^{KO} (Kim et al., 2012); *ppk12*-Gal4 (Liu et al., 2003); *ppk28*-Gal4 (Vienna *Drosophila* Resource Center [VDRC] stock number 207329); *ppk12*-Gal4 (VDRC stock number 206271); UAS-*ppk*-RNAi (VDRC stock number 108683); UAS-*ppk26*-RNAi (VDRC stock number 100834); *109(2)80*-Gal4 (Bloomington *Drosophila* Stock Center [BDSC] stock number 8768); UAS-TNTE (BDSC stock number 28837); UAS-tntV1-A (BDSC stock number 28840); *pain*^{GAL4} (BDSC stock number 27894); *w*^{1118}; UAS-mCD8::GFP; and *iav*-Gal4 (generated in our lab; data not shown).

Generation of Transgenic Flies

All the transgenic flies were generated by P-element-mediated germline transformations (Rubin, 1985) or by phi31-integrase-mediated site-specific recombinations at attP40 site (Ni et al., 2008).

The *ppk26*-Gal4 and *ppk5*-Gal4 constructs were generated by inserting the PCR-amplified promoter regions of *ppk26* and *ppk5*, respectively, into the pCasper-Aug-Gal4 vector. The *ppk26* genomic rescue construct was generated by inserting a PCR-amplified ppk26 genomic region (including promoter region, coding sequence, and 3′ UTRs) into the pCasper4 vector. UAS-*ppk26* construct was generated by inserting a PCR-amplified *ppk26* coding sequence into the pUASTattB vector.

The *ppk26*- and *rpk*-null mutants were generated by ends-out homologous recombination (Gong and Golic, 2003; Moon et al., 2009). The PCR-amplified 5′ and 3′ homologous arms were inserted into either pw35 or pw35 gal4 (Addgene plasmid 25901) vectors. The null alleles were verified by PCR and DNA sequencing.

Antibodies and Immunostaining

The following antibodies were used: rabbit anti-PPK26 antibody (against residues 497–510 [KTVINTDSPEGKEEC], obtained from Abgent; working concentration: 10 μg/ml); rabbit anti-PPK antibody (against residues 510–525 [RAFREEYEHTDAIGSR], obtained from Shanghai Youke Biotechnology; working concentration: 15 μg/ml); rabbit anti-GFP; mouse anti-GFP; Alexa 488 goat anti-rabbit immunoglobulin G (IgG); Alexa 488 goat anti-mouse IgG; biotinylated goat anti-rabbit IgG; and Rhodamine Avidin D. Antibodies were diluted in PBS- or PBS-with-Triton-blocking buffer. Fillets preparation from third instar larvae were performed as previously described and fixed in 4% paraformaldehyde solution for 20 min at room temperature. After washing, fillets were blocked in blocking buffer for 1 hr and treated with primary antibody for 2 hr at room temperature. After washing, fillets were then incubated with secondary antibody for 2 hr at room temperature. If necessary, after washing, fillets were incubated with tertiary antibody for 2 hr at room temperature and then washed.

Behavioral Assays

Flies used for behavioral tests were raised in a 25°C incubator in 12 hr dark/light cycles with a relative humidity ranging from 50% to 70%. Nociception assays were performed blind to genotypes.

Nociception Assays

The mechanical and thermal nociception assays were conducted similarly as previously described (Hwang et al., 2007; Zhong et al., 2010). Briefly, wandering third-instar larvae were tested with mechanical forces delivered by Von Frey filaments or by a silver heat probe heated to 47°C. Mechanical or thermal stimuli were delivered to the dorsal or lateral side, respectively, of the larval abdomen (segment four, five, or six). The response percentage or response delay was measured as described previously (Hwang et al., 2007; Tracey et al., 2003).

Gentle Touch Assays

The larval thoracic segments were gently touched with a fresh eyelash and the response was scored as previous described (Kernan et al., 1994).

For detailed experimental procedures, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.020.

AUTHOR CONTRIBUTIONS

Y.G., Y.W., and Z.W. designed the experiments. Q.W. and Y.W. performed the P-element-mediated germline transformation experiments. Y.G. and Y.W. performed all the other experiments and analyzed the data. Y.G., Y.W., and Z.W. wrote the paper.

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